PIGMENTED FAT CELLS IN A MUTANT OF DROSOPHILA MELANOGASTER [1]

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The "red cell" mutant of *Drosophila melanogaster* is characterized by the presence of scattered pigmented cells in the thorax and head of the adult fly. This recessive mutant factor, *rc*, is located on the second chromosome, and histological examination of the *rc* phenotype by Jones and Lewis (1957) revealed that the red pigment was localized in granules in some of the pupal fat cells. The expression of the *rc* factor was suppressed in the presence of mutant genes which interrupt the synthesis of the brown pigment of the eye: *vermilion*, *scarlet*, and *cinnabar*. On the other hand, the mutant gene *brown* which blocks the synthesis of the red component of the normal eye color did not interfere with the expression of the *rc* gene as red cells in the thorax and head of the adult fly. Therefore Jones and Lewis (1957) concluded that the pigment in the red fat cells of *rc* flies is related to the brown pigment component of the eye.

This proposed relationship to insect eye pigments enhances the usefulness of the *rc* mutant as a tool in probing the pigmentation process at a cellular level. The large pupal fat cells offer excellent material for experimental manipulations of individual cells. In addition, the implantation experiments of Beadle (1937a, 1937b) with eye discs and various tissues of *Drosophila* established the fatbody as a source of pigment precursors for the synthesis of brown eye pigment. Preliminary observations of interaction between the *rc* mutant and a mutant strain which develops melanotic masses in the fatbody suggested the present study.

Materials and Methods

A stock homozygous for both the *rc* and tu^w factors was made, and all experimental procedures utilized material from this stock. The wild type strain, *Ore-R*, was used to verify the morphological relationships of the various fat masses in the larval stages. The *rc* mutant stock was kindly provided by Dr. E. B. Lewis in 1955, and the *rc* gene has been maintained in our laboratory in combination with the tu^w factor since that time. Melanotic tumorous masses occur in larvae homozygous for the recessive factor, tu^w, located on the second chromosome. Detailed studies of this mutant stock have been reported previously (Wilson *et al.*, 1955; Rizki, 1957).

The $tu^w rc$ stock has been raised on Cream of Wheat medium with Fleischmann's yeast. Timed material for the experiments was collected in the following manner. Adult flies were placed in a half-pint bottle containing a paper teaspoon with Cream of Wheat medium heavily coated with a yeast-honey suspension. A fresh

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food spoon was placed in the bottle twice daily, and after removal from the bottle the spoons were stored in an incubator at 23°–25° C. Beginning at approximately 20 hours after a spoon had first been placed in the collection bottle, the newly emerged larvae were collected at intervals of one or two hours. These larvae were raised on Cream of Wheat-Fleischmann's yeast medium in crystallizing dishes in the incubator at 23°–25° C. All ages of larvae and pupae are thus counted from the time of eclosion from the egg.

In the starvation experiments, larvae were removed from the food dishes at 65 hours of age, rinsed in a saturated solution of NaCl, 2% solution of NaOCl, followed by repeated washing in six changes of distilled water. This procedure removed a considerable proportion of the adhering yeast and food particles. Washed larvae were placed on tissue paper strips (Kleenex brand) moistened with distilled water in petri dishes; paper was also placed under the cover of the dish to prevent larvae from crawling out of the dishes. Care was taken to maintain the paper strip moist without excessive wetting. With each starvation experiment, a group of washed larvae from the same collection period was placed in petri dishes on paper strips to which a thin layer of Cream of Wheat medium and Fleischmann's yeast was added. These larvae served as the control fed material. The larvae removed from food at 65 hours of age generally pupated several hours before those which were left on food. No difference between the percentages of adults emerging in the two groups was noted.

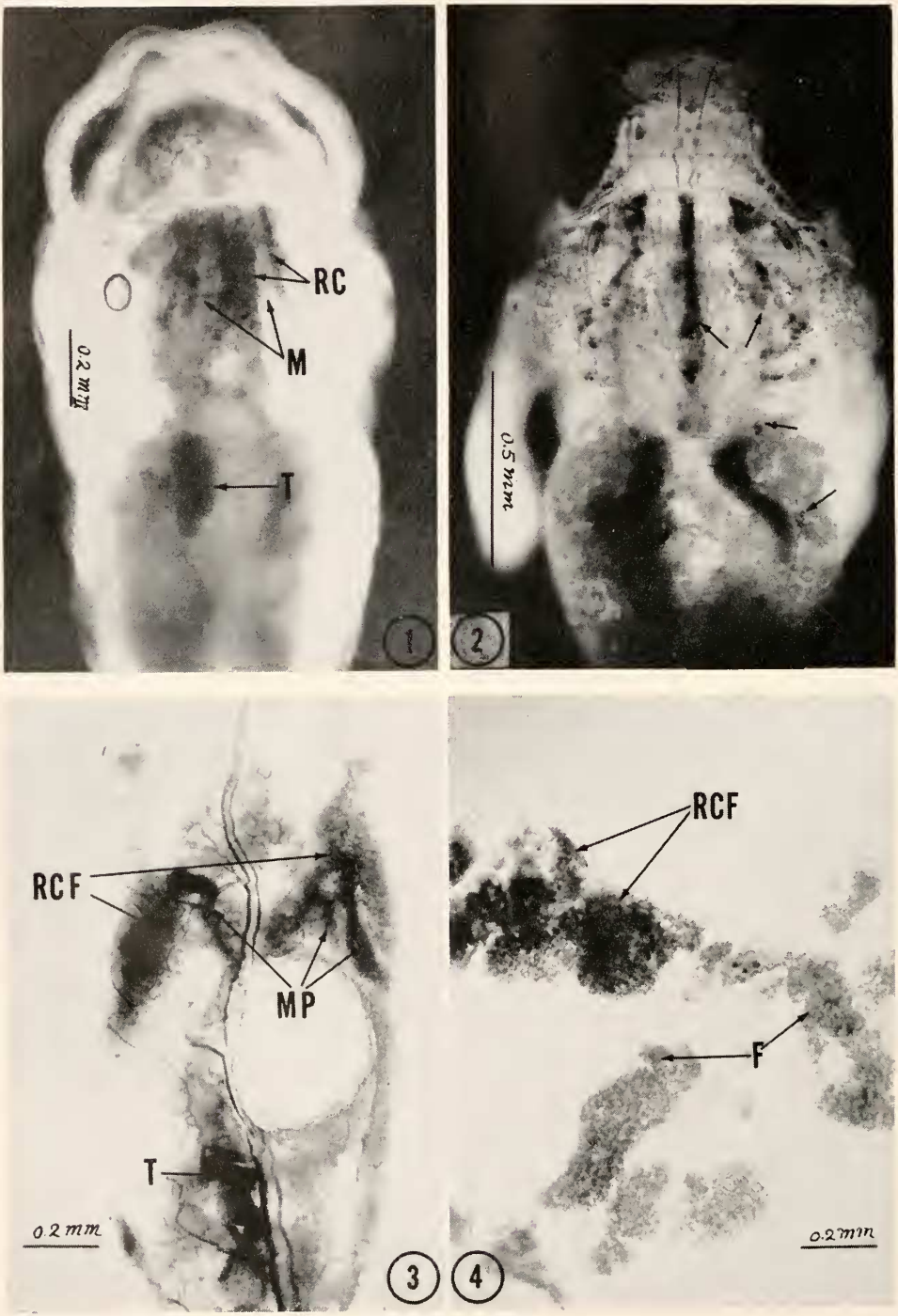
Results

No "red cells" appeared in the *tu*^{w}*rc* homozygotes in the stock bottles or in the control fed series. However, the expression of the *rc* factor was 100 per cent if the *tu*^{w}*rc* larvae were removed from food at approximately 65 hours of age, that is, during the early third instar. In this case, the mutant pattern appearing in the adult flies was the same as that described by Jones and Lewis (1957) for the *rc* stock. Red-pigmented cells were most abundant in the thorax and head, a few were found in the abdomen, and an occasional pigmented cell was seen in the appendages. In pupae shortly before hatching the red pattern in the thorax was striking. The red-pigmented cells occupied the haemocoel spaces between the flight muscles, and a dorsal striped pattern in the thorax was the result of alternating accumulations of red cells and regions of the muscle insertions (Figs. 1 and 2).

The *rc* factor exhibited no apparent influence on the development of melanotic masses in the posterior fatbody, the phenotypic character of the *tu*^{w} factor. The penetrance of the *tu*^{w} factor varied between 90% and 95% during the course of the present investigation; however, the *rc* phenotype was expressed in *tu*^{w}*rc* flies which had been starved during development whether melanotic tumors were visible or not.

Control fed larvae and starved larvae were examined at timed intervals after the beginning of the starvation period at age 65 hours. Starved larvae generally pupated two or three hours earlier than larvae which were feeding. No morphological differences were noted between the two groups of larvae until after pupation. During the early period of tanning of the puparium, a slightly yellow tinge was noticeable in the anteriormost fat masses of starved *tu*^{w}*rc* pupae. This color

PLATE I



FIGURES 1–4.

was visible through the puparium which was still very light, but removal of this covering was necessary to reveal the extent of the coloring. Figure 9 is a camera lucida drawing of a starved *tu*^{w}*rc* pupa showing the location of the yellow masses underneath the puparium, and a photograph of a specimen removed from the puparium shortly after the pupal molt is labelled Figure 3. Dissection of pupae showed that the yellow color was localized in the most anterior pair of dorsal fat masses just posterior to the cerebral hemisphere, as well as those fat masses which are lateral to this first region and extend ventrally where they join in a commissure. From the ventral side of each of these extensions, a strand of fat cells passes anteriorly where they adhere to the paired salivary glands. The fat masses just described are the only fat cells which developed the yellow pigment. Larvae of the *Orc-R* strain have been dissected to establish morphological correlations of the fat masses in the mutant strain with the fat masses in the normal material. Dissection of the larvae of the wild type strain was considerably easier since manipulation of the fat masses in the starved *tu*^{w}*rc* larvae tended to loosen the fat cells which did not seem to be held together so compactly as in the normal strain. Starvation also decreased the size of the fatbody cells as compared to fed material. In order to demonstrate the relationship of the intact fat masses, camera lucida drawings of the *Orc-R* strain are given in Figure 10. The common feature of these anterior fat masses is their proximity to the distal ends of the anterior pair of Malpighian tubules.

As pupation progressed the cells of the fatbody became separated from one another, and were thus involved in the extensive reorganization of the body tissues which takes place during metamorphosis. The pigmented cells of the anterior fatbody were redistributed during this process and were found primarily in the head and thorax of the developing pupa. The color of the fat cells in the intact fatbody of starved *tu*^{w}*rc* pupae gradually changed from yellow to yellow-brown, and as the scattering of the separated cells occurred, the color deepened, and finally red pigment was apparent. The yellow color in the fatbody appeared before the imaginal discs had everted, and the red pigment in the fat cells preceded the appearance of the red eye color.

FIGURE 1. Photomicrograph of a *tu*^{w}*rc* pupa (starved) removed from the puparium. Red cells, RC, are distributed between the longitudinal muscles, M, of the thorax. A typical melanotic tumor, T, is apparent in the abdomen and two *rc* fat cells are visible on the left margin of the tumor. (Darkfield illumination with a green filter. × 63.)

FIGURE 2. A fully formed imago removed from the puparium to show the further dispersion of the red-pigmented fat cells (arrows). Note the retention of *rc* cells in the mid-dorsal region and the further change in the distribution of *rc* cells in the areas not occupied by the insertions of the dorsoventral muscles of flight. Very few *rc* cells are seen among the fat cells of the abdominal region. The eyes are fully pigmented. (Darkfield illumination, green filter, magnification × 60.)

FIGURE 3. A *tu*^{w}*rc* (starved) prepupa removed from the pupal case, showing the position of the intact pigmented masses of fat, RCF. Note the intimate association of the anterior pair of Malpighian tubes, MP, with the RCF. The photograph represents the left dorsolateral view of the prepupa so that the left tracheal trunk is apparent and the right tracheal trunk can be visualized as a white band at the lower right margin of the photograph. The dark area under the left tracheal trunk is a melanotic tumor in the caudal fatbody, T. (Transmitted light from a Corning filter #CS 7-59. Magnification × 63.)

FIGURE 4. The intact fat masses removed from a *tu*^{w}*rc* prepupa (starved) showing the difference between pigmented fat masses, RCF, and unpigmented fat masses, F. This fat was treated with potassium metabisulfite to intensify the color. (Corning filter #CS 7-59; × 63.)

PLATE II

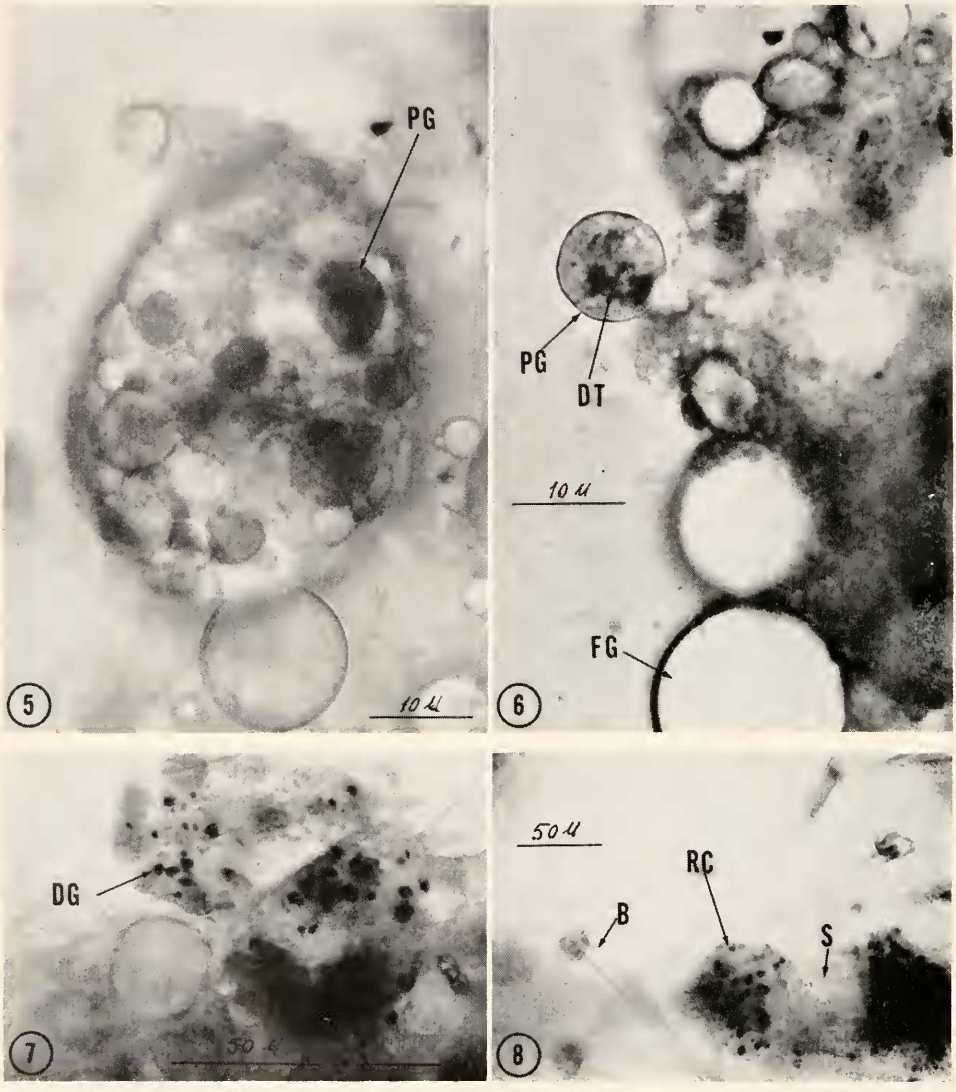


FIGURE 5. A pigmented fat cell isolated from an early prepupa (*tu*^{w}*rc* starved) showing the intracellular distribution of pigment globules, PG. (Corning filter #CS 7-59.)

FIGURE 6. A pigment globule removed from the cytoplasm of a red cell from a *tu*^{w}*rc* prepupa (starved) showing the threadlike, DT, internal structures characteristic of these globules. A fat droplet is indicated at FG. (Corning filter #CS 7-59.)

FIGURE 7. The *rc* fat cells as seen through the body wall of a *tu*^{w}*rc* starved pupa corresponding in age to that given in Figure 1. Note the appearance of pigment in granular form. (Darkfield illumination, green filter.)

FIGURE 8. Granular appearance of pigmented inclusions in a *tu*^{w}*rc* starved imago corresponding in age to that in Figure 2. Bristles, B, and setae, S, are visible in this photograph of a red cell as seen through the body wall. (Darkfield illumination, green filter.)

Both the red and brown pigment extracts from the eye of *Drosophila* are altered by oxidation and reduction (Ephrussi, 1942). It seemed desirable to determine whether the yellow pigment in the fat cells would undergo any changes *in vitro*, and a search was undertaken for conditions which might cause such an alteration. Anterior fat masses which had become yellow were dissected from starved pupae in Waddington Ringer-10% glucose. The various reagents to be tested were then added to this medium. The reducing rinse which had been prepared for use in the Feulgen reaction proved most satisfactory. It was then found

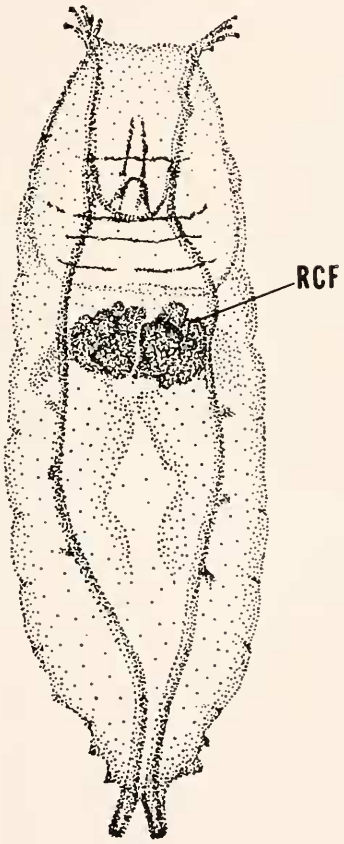


FIGURE 9. Camera lucida drawing of a dorsal view of a $tu^{w}rc$ (starved) prepupa within the puparium illustrating the position of the brownish yellow anterior fat mass, RCF.

convenient to add a few crystals of potassium metabisulfite to the drop of glucose-Ringer containing the isolated fat masses while they were under microscopic observation on a white porcelain plate. The color of the fat cells showed a change from yellow to red within a minute. Figure 4 is a photograph of the isolated fat mass in which the *rc* pigment had been intensified in this manner.

The development of pigment in the starved $tu^{w}rc$ flies has been followed at the cellular level. Isolated cells from the anterior fatbodies of young pupae contained

numerous yellow cytoplasmic globules. These globules were distinguishable from the fat droplets of the cells which are highly refractile and always spherical in fresh preparations. Isolated cells have been examined with darkfield illumination as well as brightfield illumination, and in addition, the use of a blue Corning

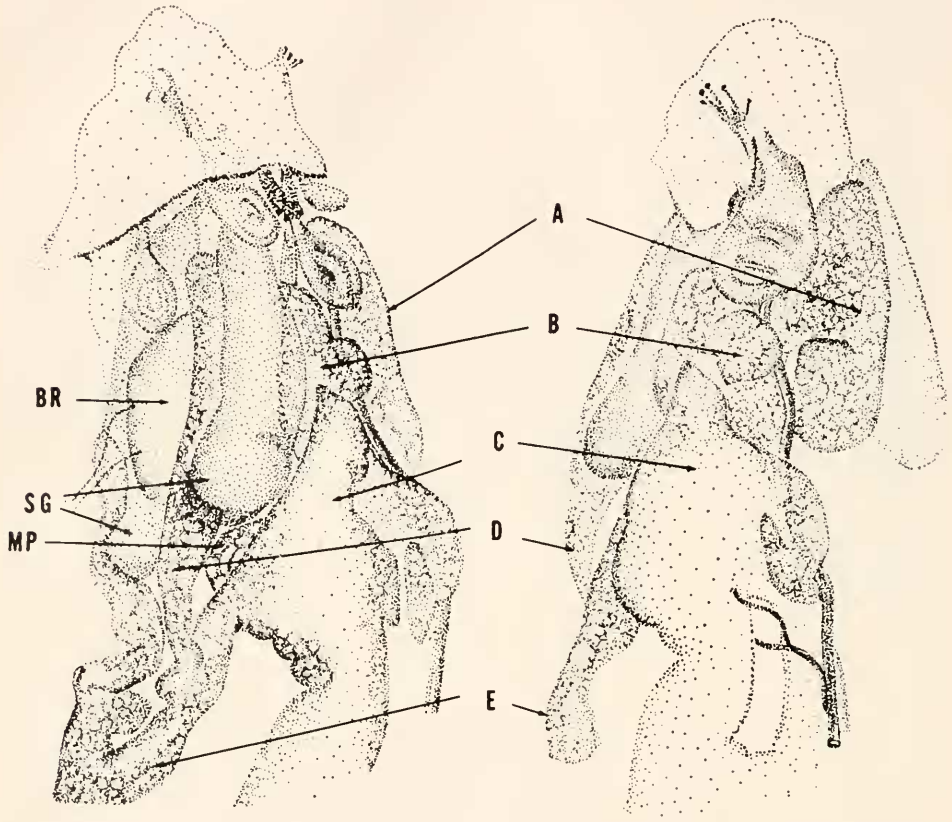


FIGURE 10. Camera lucida drawings of a dissection of a late third instar larva of the *Ore-R* strain showing the relative positions of the fat masses; the salivary glands, SG; brain, BR; Malpighian tubes, MP. This specimen has been stained with Oil Red O. The diagram on the left is the ventrolateral view, and the same dissection has been turned over to show the lateral view of the fat masses in the drawing on the right. A, B, C, D, E are the regions of the fatbody which become pigmented in starved *tu*^{w}*rc* pupae. A, dorsal pair of fat masses; B, C, lateral fat masses; D, fat mass attached to salivary gland; E, fat cells forming the ventral commissure between the right and left masses of fat. Only the anterior region of C becomes pigmented. The club-shaped structures with concentric rings are the imaginal discs and the long tubular structure with branches is the tracheal trunk.

Filter No. CS 7-59 proved most satisfactory for studying the cytoplasmic inclusions in the fat cells. With this filter, all yellow objects appeared bright red. In early *tu*^{w}*rc* pupae (after starvation), this included the lightly tanned cuticle, isolated cells from melanotic masses, the granular structures in the Malpighian tubules,

and the yellow globules in the *rc* fat cells. The yellow globules showed a definite threadlike internal structure when examined with this filter, while no structure was discernible in the fat droplets (Figs. 5 and 6). Fat cells from the more posterior regions of the pupa, *i.e.*, cells other than the fatbody cells involved in the expression of the *rc* phenotype, contained similar cytoplasmic inclusions in addition to the fat droplets. Whether these inclusions are structurally and functionally the same as those globules which become pigmented in the *rc* cells remains to be examined. The cells containing the yellow globules were placed on a slide in Waddington-10% glucose solution and several crystals of potassium metabisulfite were added under the coverslip in the vicinity of the cells. The yellow inclusions became red in color under these conditions.

After the pigmented fat cells had become scattered throughout the thorax and head during the developmental processes occurring in pupal life, the color in the fat cells appeared more intense. In the late pupae and young adults, the pigmented structures in these cells were more granular in appearance (Figs. 7 and 8).

Discussion

Two types of pigment are found in the eye of *Drosophila*, one brown and the other red, and many mutants are known which affect the production of these pigment components in the eye. Interference with brown pigment production results in a bright red eye color of the type found in the mutants *vermilion*, *cinnabar*, and *scarlet* while the phenotype of the *brown* eye mutant represents an interruption in the biochemical pathways leading to red pigment. The absence of both pigments occurs in the mutant, *white* eye. The literature on brown eye pigments in insects has been reviewed by Ephrussi (1942) Nolte (1952), and Kikkawa (1953). The synthesis of brown pigment proceeds through a pathway involving tryptophan, formylkynurenine, kynurenine, and hydroxykynurenine, and the known eye color mutants are blocks at successive stages in this synthetic chain. Transplantation of imaginal discs of mutant larvae into hosts of different genotypes has shown that in some cases the eye color is autonomous, whereas some mutant eyes do not themselves produce the prerequisites for brown pigment and are dependent upon other sources in the body for these precursors (Beadle and Ephrussi, 1936; Ephrussi, 1942; Ephrussi and Beadle, 1937). The presence of these pigment precursors has been demonstrated in the fatbody and the Malpighian tubules by transplantation experiments (Beadle, 1937a, 1937b). The time during which each of these tissues produced the pigment precursors was dependent upon the stage of development of the donor. Malpighian tubes showed activity through larval life from the earliest stages tested, appearance of active substances in the fatbody was not detected until after pupation, and in the eyes much later during pupal development (Beadle, 1937b; Clancy, 1940).

The pigment granules in the *rc* cells have been shown to be related to the brown eye pigment of *Drosophila*. Jones and Lewis (1957) found that the mutant factors, *vermilion*, *cinnabar* and *scarlet*, which interfere with brown pigment development in the eye, also prevent the formation of pigment in the *rc* cells when each of these mutant factors is combined with the *rc* gene. The mutant factor, *brown*, which blocks the synthesis of red pigment, does not interfere with the expression of the *rc* gene in the fat cells.

The explanation for the suppression of pigment in the fat cells when the *rc* gene is combined with the tu^{w} factor may not be so direct. In the starvation experiments the penetrance of the tu^{w} factor was 90%–95%, and no difference in the expression of *rc* was noted between the pupae with melanotic tumors and those that did not develop black masses. One point of comparison is the fact that both mutants have a common domain of expression, *i.e.*, fat cells: *rc*, the anterior fat mass and tu^{w}, the posterior region of the fatbody. Tryptophan metabolism is not only related to the development of brown eye pigment and protein metabolism, but it also influences the expressivity and the penetrance of various melanotic tumor genes in *Drosophila*. Addition of tryptophan to the medium increases the frequency of melanotic tumors in strains carrying tumor genes (Hartung and Hartnett, 1951; Plaine and Glass, 1955), and Kanehisa (1956a, 1956b) reported an increase in tumor incidence by combining a tumorous factor with eye color genes. The appearance of pigmented fat cells in $tu^{w}rc$ pupae after starvation parallels the behavior of the *vermilion* mutants which develop brown eye pigment after the larvae have been starved (Beadle *et al.*, 1938). Starvation of *Drosophila* adults results in a reduction in the size of the fatbody, and the reserves of fat and glycogen are rapidly depleted from the fat cells (Wigglesworth, 1949). In many insects the fatbodies may serve as storage sites for excretory products as well as food reserves. Wigglesworth (1942) has shown that starvation of *Aëdes* larvae causes an increase in uric acid vacuoles in the fat cells and these deposits disappear from the cells after the feeding has resumed. The conditions imposed by starvation in the $tu^{w}rc$ larvae alter the metabolic pattern of the fat cell such that it differentiates as a pigmented cell. A similar effect, of course, is produced in the *rc* mutant under normal feeding conditions. The presence of the tu^{w} factor may restore the normal metabolic balance in the fat cell such that their phenotype resembles that of the wild type. The expression of the *rc* phenotype is also dependent upon the action of another recessive gene, *lys*, which causes an accumulation of the amino acid, lysine (Grell, 1958). It is thus obvious that the expression of the red cell phenotype is influenced by the interaction of a number of non-allelic genes. One suggestion may be made which will encompass the various aspects of the problem known at the present time. Any modification, genetic or environmental, which influences the normal pattern of protein synthesis will also alter the metabolic pool of various amino acids. Such changes which affect the availability of tryptophan may be reflected in the phenotypic expression of the *rc* pigment.

The larval fat cells of *Drosophila* form organized tissue masses, whereas soon after pupation the fatbody becomes separated into single cells or small clusters of cells. The cells of the caudal fat masses in tu^{w} larvae which are involved in the production of melanotic tumors in this strain resemble pupal fat cells in their tendency toward smaller cell aggregations and a loss of adherence to neighboring cells (Rizki, 1957). This precocious change in the structure of the caudal fatbody of tu^{w} larvae, as well as precocious changes in the blood cells, are processes which lead to tumor formation in the caudal fatbody prior to pupation. Therefore the hypothesis was presented that the melanotic masses in this tumorous strain of *Drosophila* represented an upset in the normal timed pattern of events occurring during metamorphosis. Under conditions favoring expression of the *rc* gene, the

tu^{w} factor influences an earlier appearance of red pigment in the anterior fat cells. This pigmentation in the *rc* mutant is not apparent until after the fat cells become isolated and scattered during the pupal stage (Jones and Lewis, 1957). However, the combination of *rc* with the *tu*^{w} factor has shifted the time of development of the red pigment to a stage preceding this dispersion of the fat cells. Although no obvious explanation for the distribution of the red fat cells among other non-pigmented fat cells existed in the *rc* strain, the morphological relationship of all the red cells in larval development becomes apparent in the *tu*^{w}*rc* starved material. A measure of the dispersion of the cells of the anterior fat masses during early pupation is provided in this case by a mutant cytoplasmic marker. The localization of mutant characteristics in the *tu*^{w} caudal fat masses and the anterior *rc* fat cells suggests that the cells of various regions of the fatbody may differ in their developmental physiology. It is interesting to note that the cellular ecology of the anterior and the posterior fatbody includes a common feature: the *rc* fat cells are intimately associated with the distal ends of the anterior pair of Malpighian tubules, and the *tu*^{w} fat cells encircle the distal ends of the posterior Malpighian tubules.

Summary

1. Cytoplasmic pigment granules are found in some of the fat cells of the recessive mutant, *rc*, of *Drosophila melanogaster*. These scattered red fat cells are located chiefly in the thorax and head, but a few occur in the abdomen and appendages of the adult. Using genetic methods it had been shown previously that these pigment accumulations are related to the synthesis of the brown eye pigment of this insect (Jones and Lewis, 1957).

2. The pigmentation in the *rc* fat cells is suppressed when the *rc* gene is combined with the recessive factor, *tu*^{w}. This combination, however, in no way alters the expression of the characteristic pattern of *tu*^{w} as revealed by the presence of melanotic tumors in the caudal fat masses of the homozygous *tu*^{w}*rc* flies. After a period of larval starvation, the *tu*^{w}*rc* flies develop both the red-pigmented fat cells and melanotic tumors. The time of appearance of the *rc* pigment has been shifted under these nutritional and genetic conditions. The cytoplasmic pigment granules appear in the cells originating from the anteriormost section of the larval fatbody which is closely associated with the anterior Malpighian tubules. During the reorganization accompanying metamorphosis from the larval to the adult stage, these cells are redistributed mostly to the thoracic and cephalic regions while a few are found in the abdomen and appendages. An explanation is thus provided for the cytodifferentiation of pigmented and nonpigmented fat cells found side by side in the adult fly.

3. The nature of the pigment granules has been examined in *in vitro* preparations at each of these periods of development, and of particular interest is the internal threadlike structure of these cytoplasmic inclusions during the early stages of pigment formation.

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